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Bispecific antibody approach for improved melanoma-selective PD-L1 immune checkpoint blockade.

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Short title: Melanoma-directed blockade of PD-L1

Abbreviations: CSPG4, Chondroitin Sulfate Proteoglycan 4; Programmed cell Death protein 1 (PD-1); PD-L1, programmed death receptor ligand-1; bsAb, bispecific antibody; TILs, tumor-infiltrating lymphocytes; IFN-γ, interferon-γ.

ABSTRACT

Reactivation of functionally-impaired anticancer T cells by PD-1/PD-L1-blocking antibodies shows prominent therapeutic benefit in advanced melanoma and NCSLC patients. However, current PD-L1-blocking antibodies lack intrinsic tumor-selectively. Therefore, efficacy may be reduced due to 'on-target/off-tumor' binding to PD-L1-expressing normal cells. This may lead to indiscriminate activation of antigen-experienced T cells, including those implicated in autoimmune-related adverse events. To direct PD-L1-blockade to CSPG4-expressing cancers and to reactivate anticancer T cells more selectively we constructed bispecific antibody (bsAb) PD-L1xCSPG4. CSPG4 is an established target antigen that is selectively overexpressed on malignant melanoma and various other difficult-to-treat cancers. PD-L1xCSPG4 showed enhanced capacity for CSPG4-directed blockade of PD-L1 on cancer cells. Importantly, treatment of mixed-cultures containing primary patient-derived CSPG4-expressing melanoma cells and autologous tumor-infiltrating lymphocytes with PD-L1xCSPG4 significantly enhanced activation status, IFN-γ production, and cytolytic activity of anticancer T cells. In conclusion, tumor-directed blockade of PD-L1 by PD-L1xCSPG4 may improve efficacy and safety of PD-1/PD-L1 checkpoint blockade for treatment of melanoma and other CSPG4-overexpressing malignancies.

INTRODUCTION

Malignant melanoma is the most lethal type of skin cancer and its incidence is rising at an alarming rate of approximately 1.5% annually over the last decade (National Cancer Institute, 2018). When diagnosed at an early stage, localized melanoma can be treated by radical removal of the lesion, resulting in excellent survival rates. However, once progressed to the metastatic stage, the options for curative treatments are limited. Recently, immune checkpoint PD-1-blocking antibodies nivolumab and pembrolizumab have shown remarkable benefit for a subgroup of patients suffering from metastatic cancer, including malignant melanoma (Sullivan and Flaherty, 2015, Wolchok et al, 2017). However, systemic administration of both PD-1 and PD-L1-blocking antibodies carries the potential risk of inducing serious immune-related adverse events (irAEs) (Pen et al, 2014). Moreover, the efficacy of current PD-1/PD-L1-blocking antibodies may be reduced due to on-target/off-tumor binding to a surplus of normal cells also expressing PD-L1, which may preclude sufficient antibody accumulation at the tumor site..

Therefore, we developed a bispecific antibody which aims to locally reactivate anticancer T cells by directing PD-L1-blockade to CSPG4 expressed on the surface of tumor cells. Chondroitin sulfate proteoglycan 4 (CSPG4), also known as MCSP, NG2 (Nishiyama et al, 1991) and HMW-MAA (Wilson, Ruberto and Ferrone, 1983) is a type 1 transmembrane protein that consists of two components - an N-linked 280 kDa glycoprotein component and a 450 kDa chondroitin sulfate proteoglycan component. CSPG4 is selectively overexpressed on several difficult-to-treat cancer types, including melanoma, mesothelioma (Rivera et al, 2012), triple-negative breast cancer (TNBC) (Wang et al, 2010), and glioblastoma (Svendsen et al, 2011). Importantly, more than 90% of melanoma lesions overexpress CSPG4 (Campoli et al, 2004). Consequently, CSPG4 is considered a promising target antigen for antibody-based therapy of these malignancies (reviewed in (Jordaan et al, 2017)). The remarkable

cancer-selective expression of CSPG4 prompted us to construct a bsAb that allows for CSPG4-directed blockade of the PD-1/PD-L1 immune checkpoint. This targeted approach may be a next step in enhancing efficacy and safety of PD-1/PD-L1 checkpoint inhibition in CSPG4-overexpressing cancers.

RESULTS

PD-L1xCSPG4 binds to both PD-L1 and CSPG4

PD-L1xCSPG4 showed potent and dose-dependent binding to CHO.PD-L1 hamster cells ectopically expressing human PD-L1 and essentially no binding to PD-L1^{neg} wt CHO cells (Fig. 1a). Importantly, PD-L1xCSPG4 showed enhanced binding to CSPG4^{pos}/PD-L1^{pos} MDA-MB-231.CSPG4+++ breast cancer cells compared to CSPG4^{low}/PD-L1^{pos} wt MDA-MB-231 cells (Fig. 1b). Furthermore, binding of PD-L1xCSPG4 to CSPG4^{pos}/PD-L1^{pos} A375m melanoma cells was strongly reduced in the presence of excess amounts of the parental anti-CSPG4 antibody mAb 9.2.27, whereas presence of excess amounts of a competing PD-L1-blocking mAb only marginally inhibited cancer cell binding (Fig. 1c). Thus, PD-L1xCSPG4 binds to both PD-L1 and CSPG4 and its binding to CSPG4^{pos}/PD-L1^{pos} melanoma cells is dominated by binding to overexpressed CSPG4. Of note, binding data of PD-L1xCSPG4 to a series of PD-L1^{pos}/CSPG4^{pos} cell lines closely correlated with expression levels of CSPG4 on the respective cell lines (Fig. 1d and e), whereas binding of PD-L1xMock, a PD-L1-blocking bsAb equipped with an irrelevant second binding specificity, was correlated only with PD-L1 expression levels (suppl. Fig. 2a-c).

The enhanced binding affinity (avidity) of PD-L1xCSPG4 for CSPG4^{pos}/PD-L1^{pos} cancer cells was evaluated in a competitive cell binding assay with fluorescently-labeled anti-PD-L1 mAb. Using this assay, the IC50 value of PD-L1xCSPG4 for displacing an APC-labeled PD-L1 mAb bound to A375m melanoma cells was calculated to be 4.6 ng/ml, ~100 times lower than that of control bsAb PD-L1xMock. Importantly, in the presence of a molar excess of CSPG4-competing mAb 9.2.27 the IC50 value of PD-L1xCSPG4 increased to that of PD-L1xMock (Fig. 1f).

Taken together, these data demonstrated that PD-L1xCSPG4 has markedly enhanced avidity for cancer cells that express both CSPG4 and PD-L1.

CSPG4-directed PD-1/PD-L1 blockade by PD-L1xCSPG4

CSPG4-independent PD-L1-blocking capacity of PD-L1xCSPG4 was evaluated using a commercially available bioassay in which antibody-induced release of the PD-1/PD-L1-mediated break on luciferase production in Jurkat.PD1-NFAT-luc cells by CHO.PD-L1/CD3 cells is evaluated by an increase in bioluminescence. In this assay, PD-L1xCSPG4 and PD-L1xMock dose-dependently inhibited PD-1/PD-L1 interaction with similar IC50 values of 2.07 and 2.99 µg/ml, respectively. Notably, the PD-L1-blocking activity of antibody MEDI4736 proved to be significantly higher (IC50 = 0.09 µg/ml) (Fig. 2a).

Next, the capacities for CSPG4-directed PD-1/PD-L1 blockade of PD-L1xCSPG4, PD-L1xMock and MEDI4736 were compared using an adapted version of the aforementioned PD-1/PD-L1 bioassay by exchanging CHO.PD-L1/CD3 cells with A375m.EpCAM melanoma cells stably transfected with EpCAM and pretreated with BIS-1; an EpCAM-directed CD3-agonistic bsAb, essentially as previously described by us (Kroesen et al, 1997, Koopmans et al, 2018). PD-L1xCSPG4 showed an enhanced capacity to unleash the PD-1/PD-L1-mediated break on luminescence by Jurkat.PD1-NFAT-luc cells compared with PD-L1xMock (Fig. 2b). However, when binding to CSPG4 on A375m.EpCAM melanoma cells was precluded by pretreatment with parental anti-CSPG4 antibody mAb 9.2.27, the ability of PD-L1xCSPG4 to block PD-1/PD-L1 interaction was reduced to that of PD-L1xMock (Fig. 2c).

Together, this indicates that the PD-L1-blocking capacity of PD-L1xCSPG4 towards $CSPG4^{neg}$ cells is notably lower than that of MEDI4736 and comparable to that of PD-L1xMock. However, upon CSPG4-binding the PD-L1-blocking activity of PD-L1xCSPG4 increases to that of MEDI4736 and outperforms that of PD-L1xMock.

PD-L1xCSPG4 promotes activation status of antigen-experienced T cells

In a mixed lymphocyte reaction (MLR) of CFSE-labeled PBMCs and allogeneic DCs, treatment with PD-L1xCSPG4,PD-L1xMock or MEDI4736 enhanced the capacity of T cells to proliferate and secrete IFN-γ (Fig. 3a and b). Subsequently, we evaluated PD-L1xCSPG4 for its capacity to enhance the activity of antigen-experienced T cells. For this, PBMCs derived from CMV-seropositive and CMV-seronegative healthy subjects were incubated with recombinant CMV protein pp65 and then cultured in the presence of PD-L1xCSPG4 or control antibodies. Of note, incubation of PBMCs with pp65 protein results in phagocytic processing and subsequent cross-presentation of pp65-derived peptides in the context of the respective autologous HLA-class 1 haplotype. Treatment of pp65-loaded PBMCs with PD-L1xCSPG4 or MEDI4736 increased the secretion of IFN-γ and Granzyme B by autologous T cells derived from CMV-seropositive subjects, but not from CMV-seronegative subjects (Fig. 3c and d). Taken together, like MEDI4736, PD-L1xCSPG4 and PD-L1xMock have capacity to enhance proliferation, Granzyme B and IFN-γ secretion by antigen-experienced T cells.

PD-L1xCSPG4 enhances anticancer activity of T cells in a CSPG4-directed manner

Next, we assessed the ability of PD-L1xCSPG4 to promote anticancer activity of T cells in a CSPG4-directed manner. First, a treatment regime was applied in which mixed cultures of T (effector) cells and $CSPG4^{pos}$ A375m melanoma (target) cells were treated in the continuous presence of PD-L1xCSPG4 or control antibodies for 48 h, after which induction of IFN-γ production by T cells was detected by an ELISpot assay. Under this regime, the amount of IFN-γ spots induced by PD-L1xCSPG4 was higher than induced by the various control antibodies (Fig. 4a and b).

Subsequently, we evaluated a treatment regime in which T cells were mixed with either $CSPG4^{pos}$ (A375m and SK-MEL-28) or $CSPG4^{neg}$ cancer cells (FaDu) and treated with PD-

L1xCSPG4, PD-L1xMock or MEDI4736 for only 1 h, after which unbound antibody was removed and treatment was allowed to continue for 48 h. Under this regime, PD-L1xCSPG4 showed enhanced capacity to induce T cell produced IFN-γ spots when cocultured with CSPG4^{pos} A375m and SK-MEL-28 cancer target cells, but not with CSPG4^{neg} FaDu cancer cells (Fig. 4c). Of note, the inter-donor variation in (normal) immune responses, e.g. in individual IFN-γ response levels, is reflected in the variation observed in the amount of IFN-γ ELIspots as detected for the individual donors in Fig. 4a.

PD-L1xCSPG4 enhances anticancer activity of TILs towards autologous patient-derived melanoma cells

Primary melanoma cells and autologous tumor-infiltrating lymphocytes derived from 5 patients were short-term cultured *in vitro.* The melanoma cells were assessed for cell surface expression of CSPG4 en PD-L1 by flow cytometry, which revealed that 5 out of 5 tumor specimens expressed both CSPG4 and PD-L1 (Fig. 5a and b). Importantly, in coculture experiments PD-L1xCSPG4 enhanced the anticancer activity of TILs towards autologous patient-derived melanoma cells, which was evident from an up to 25% increase in apoptotic cancer cell death compared to medium control (Fig. 5c). Moreover, MEDI4736 also enhanced the anticancer activity of TILs towards autologous patient-derived melanoma cells up to 10%, whereas MockxCSPG4 did not. The increase in oncolytic activity of TILs by PD-L1xCSPG4 and MEDI4736 treatment was accompanied by an increase in their capacity to express CD25 (Fig. 5d) and secrete IFN-γ (Fig. 5e). Taken together, these results indicate that PD-L1xCSPG4 enhances activation status and anticancer activity of TILs towards autologous patient-derived melanoma cells. Of note, CSPG4 expression on patient-derived melanoma cells was evaluated by flow cytometry using a primary FITC-conjugated anti-CSPG4 mAb

without further signal amplification to minimize handling steps. Consequently, the CSPG4 expression levels as shown in Fig. 5 are in fact underestimated (see suppl. Fig. 5 a-d).

DISCUSSION

Current FDA-approved PD1/PD-L1-blocking antibodies show prominent therapeutic activity, particularly in advanced NSCLC and melanoma patients. However, these antibodies lack tumor-selective binding activity and may indiscriminately reactivate antigen-experienced T cells, including potentially harmful autoreactive T cells. Recently, we reported that NSCLC-directed PD-1/PD-L1 blockade can be significantly enhanced through the use of bsAb PD-L1xEGFR that directs PD-L1-blockade to EGFR-overexpressing cancer cells *in vitro* and shows enhanced tumor-selective localization in nude mice xenografted with EGFR-positive tumors (Koopmans et al, 2018).

For the current study, we extended this approach to melanoma cells by constructing bsAb PD-L1xCSPG4. CSPG4 appears a promising target antigen for this approach because of its overexpression on more than 90% of melanoma lesions and its absence on adult healthy tissues. Moreover, CSPG4 is implicated in various malignant features of melanoma. In particular, CSPG4 signaling stimulates growth, motility, and tissue invasion by melanoma cells, e.g. by enhancing integrin function (Eisenmann et al, 1999), activation of Focal Adhesion Kinase (FAK) (Yang et al, 2004), mitogenic ERK signaling (Yang et al, 2009) and matrix metalloproteinase 2 (Iida et al, 2007). Additionally, CSPG4 is also expressed on various other difficult-to-treat malignancies, including mesothelioma (Rivera et al, 2012), triple-negative breast cancer (TNBC) (Wang et al, 2010), and glioblastoma (Svendsen et al, 2011).

Our binding analyses demonstrated that PD-L1xCSPG4 simultaneously binds to PD-L1 and CSPG4 and that this concurrent binding enhances avidity of binding to PD-L1^{pos}/CSPG4^{pos} cancer cells. Of note, bsAb PD-L1xCSPG4 not only binds with enhanced affinity to cancer cells that express both CSPG4 and PD-L1, but can also can bridge two different cell types that express either of these target antigens and thereby modulate intercellular contacts (see suppl. Fig. 3 a and b). The latter may further add to the tumor-directed activity of PD-L1xCSPG4 as it may locally block the immune suppressive activity of tumor-infiltrating leukocytes such as myeloid-derived suppressor cells (MDSCs) that are known to exert their suppressive action by expressing elevated cell surface levels of PD-L1 in the tumor microenvironment.

Using a modified bioassay, we demonstrated that the enhanced avidity of PD-L1xCSPG4 for PD-L1^{pos}/CSPG4^{pos} cancer cells resulted in strong PD-L1-blocking activity, similar to that of high-affinity PD-L1-blocking antibody MEDI4736. Importantly, compared to MEDI4736 the PD-L1-blocking activity of PD-L1xCSPG4 towards PD-L1^{pos}/CSPG4^{neg} cells was clearly lower. Thus, the PD-L1-blocking capacity of PD-L1xCSPG4 is increased to that of MEDI4736, but only upon concurrent binding to cancer cell surface-expressed CSPG4. This unique feature of PD-L1xCSPG4 may be attributable to its particular bispecific taFv-Fc format in which the carboxyl terminus of each PD-L1-blocking scFv antibody domain is fused to the amino terminus of each CSPG4-directed scFv antibody domain, interspersed by only a short linker sequence (suppl. Fig. 1a). Previously, it was demonstrated that linker composition and/or length used in this class of bsAbs can reduce accessibility to either or both target antigens (Piccione et al, 2015, Wu et al, 2009).

Our results indicated that, like MEDI4736, PD-L1xCSPG4 promotes the activation status of antigen-experienced T cells. In particular, treatment of pp65 peptide-presenting PBMCs with PD-L1xCSPG4 promoted the capacity of autologous T cells derived from CMV-seropositive

subjects, and not from CMV-seronegative subjects, to proliferate and secrete IFN-γ and Granzyme B.

Importantly, our data indicated that PD-L1xCSPG4 promotes the activation status of T cells towards cancer cells in a CSPG4-directed manner. This unique feature of PD-L1xCSPG4 was particularly prominent in a regime in which mixed cultures of T cells with either $CSPG4^{pos}$ or $CSPG4^{neg}$ cancer cells were briefly treated for 1 h, after which unbound antibody was washed away. Under this regime, PD-L1xCSPG4 enhanced the capacity of T cells to produce IFN-γ, but only when cocultured with $CSPG4^{pos}$ melanoma cells and not with $CSPG4^{neg}$ cells. Moreover, PD-L1xCSPG4 enhanced the activation status and anticancer activity of TILs towards corresponding autologous patient-derived $PD\text{-}L1^{pos}/CSPG4^{pos}$ melanoma cells.

Apart from the above characteristics, PD-L1xCSPG4 may also have other anticancer activities not addressed here. For instance, it is reported that the anti-CSPG4 mAb 9.2.27 used to construct PD-L1xCSPG4 has capacity to inhibit anchorage-independent growth of human melanoma cells (Harper and Reisfeld, 1983). It is tentative to speculate that this activity is retained in bsAb PD-L1xCSGP4 and that concurrent blockade of PD-L1 may result in further sensitization of melanoma cells to therapy. Additionally, PD-L1xCSPG4 is equipped with a human IgG1 domain which may be beneficial to selectively eliminate $PD\text{-}L1^{pos}/CSPG4^{pos}$ cancer cells by NK cell-mediated ADCC. Indeed, it was recently demonstrated that PD-L1-blocking avelumab, engineered with a human IgG1 domain, has NK cell-mediated ADCC activity that enhanced its therapeutic functionality with a toxicity profile similar to ADCC-null mutated PD-L1-blocking antibodies (Boyerinas et al, 2015, Fujii et al, 2016).

Taken together, bispecific antibody PD-L1xCSPG4 may be useful to enhance selectivity, efficacy and safety of PD-1/PD-L1 checkpoint blockade for treatment of melanoma and other CSPG4-overexpressing malignancies. Further development of this approach appears warranted.

MATERIALS AND METHODS

Antibodies and Reagents

Goat anti-human Ig-PE (Southern Biotech, Birmingham, AL), anti-PD-L1-APC (clone 29E.2A3, BioLegend, San Diego, CA), anti-CSPG4-FITC (clone LHM2, Santa Cruz Biotechnologies, CA), anti-CSPG4-PE (clone 9.2.27, BD Biosciences, Franklin Lakes, NJ), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, all eBioscience, Waltham, MA), and anti-CD3-FITC (clone Ucht1), anti-CD8-FITC, APC (clone HIT8a), anti-CD56-PE (clone B-A19), anti-CD14-FITC, PE (clone MEM-15), anti-CD25-FITC, APC (clone MEM-181), mouse IgG1-FITC, PE, Mouse IgG2b-APC, Annexin-V-FITC (all Immunotools, Friesoythe, Germany). CSPG4-blocking mAb was from BioLegend, clone 9.2.27. PD-L1-blocking mAb was from BPS Bioscience (San Diego, CA). Secretion of cytokines by T cells was measured using appropriate ELISA kits (IFN-γ from eBioscience and granzyme B from Mabtech, Nacka Strand, Sweden).

Cell lines and transfectants

Cell lines A2058, A375m, G43, SK-MEL-28, HT1080, MDA-MB-231, FaDu, H292, LNCaP, 22Rv1 and CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were authenticated by short tandem repeat (STR) analysis and regularly checked for mycoplasma infection. Cells were cultured in RPMI-1640 or DMEM (Lonza, Bazel, Swiss), supplemented with 10% fetal calf serum (FCS, Thermo Scientific Waltham, MA), CHO-K1 cells were cultured in GMEM (First Link, Wolverhampton, UK), supplemented with 5% dialyzed FBS (Sigma Aldrich) at 37°C in a humidified 5% CO_2 atmosphere. CHO.PD-L1 cells stably expressing human PD-L1 were generated by lipofection (Fugene-HD, Promega, Madison, WI) with plasmid pCMV6-PD-L1 (Origene, Rockville MD). A375m cells stably expressing EpCAM were generated by lipofection with plasmid

EpCAM-YFP (a kind gift from Dr. Olivier Gyres, Munich, Germany). Cell line MDA-MB-231.CSPG4+++ (Ilieva et al, 2018) was a kind gift from Dr. Sophia N. Karagiannis (King's college Londen, United Kingdom). CSPG4 and PD-L1 expression was analyzed for all cell lines by flow cytometry using anti-CSPG4-PE and anti-PD-L1-APC antibodies and appropriate isotype controls. The relative expression levels of CSPG4 and PD-L1 are listed in suppl. Table 1.

Construction of bsAb PD-L1xCSPG4

DNA fragments encoding scFvPD-L1 and scFv 9.2.27 were generated by commercial gene synthesis service (Genscript, Piscataway, NJ) based on published VH and VL sequence data of PD-L1-blocking antibody 3G10 and CSPG4-directed mAb 9.2.27, respectively. For construction and production of PD-L1xCSPG4 we used eukaryotic expression plasmid pEE14-bsAb (He et al, 2016), which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 IgG domain (Helfrich et al, 2000). MCS#1, MCS#2 and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFv9.2.27, and human Fc (IgG1), respectively, yielding plasmid pEE14-PD-L1xCSPG4 (suppl. Fig. 1a and b).

SDS-PAGE analysis of PD-L1xCSPG4

Protein A-purified PD-L1xCSPG4 or MEDI4736 (2,5µg) were separated by SDS-PAGE (10% acrylamide) under reducing or nonreducing conditions, followed by staining of the gel with Coomassie Brilliant Blue (suppl. Fig. 1c).

Eukaryotic production of recombinant bsAbs

PD-L1xCSPG4 was produced using CHO-K1 cells transfected with eukaryotic expression plasmids pEE14-PD-L1xCSPG4, using the Fugene-HD reagent (Promega) and stable transfectants were generated by the glutamine synthetase selection method. Stable transfectants were cultured at 37°C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies, Waltham, MA) for up to 7 days after which supernatant was harvested (3000 x g, 30 min). PD-L1xCSPG4 was purified using a HiTrap protein A HP column connected to an ÄKTA Start chromatography system (GE Healthcare Life Sciences, Little Chalfont, UK).

Binding activity of PD-L1xCSPG4 for PD-L1 and CSPG4

PD-L1-selective binding activity of PD-L1xCSPG4 was confirmed by comparing binding to CHO.PD-L1 cells versus parental CHO cells. Similarly, CSPG4-selective binding activity of PD-L1xCSPG4 was confirmed by comparing binding to MDA-MB-231.CSPG4+++ cells versus parental $CSPG4^{low}$ MDA-MB-231 cells. In short, cancer cells were incubated with increasing concentrations of PD-L1xCSPG4 (0.001-10 µg/ml) at 4°C for 45 min, washed, incubated with anti-human-Ig-PE mAb and evaluated by flow cytometry. Additionally, binding selectivity of PD-L1xCSPG4 for endogenously expressed CSPG4 was assessed using $CSPG4^{pos}$ cancer cell lines A375m, G43, SK-MEL-28, A2058, HT1080, MDA-MB-435 versus $CSPG4^{neg}$ cancer cell lines H292, FaDu, LNCaP, 22Rv1 by flow cytometry.

Competitive binding assay

The overall binding strength (avidity) of PD-L1xCSPG4, PD-L1xMock, and MockxCSPG4 for $PD\text{-}L1^{pos}$/$CSPG4^{pos}$ cancer cells was compared in a competitive binding assay. In short, A375m melanoma cells were pre-incubated (or not) with excess amounts of mAb 9.2.27 (50

µg/ml) at 4°C for 45 min, after which PD-L1xCSPG4, PD-L1xMock or MockxCSPG4 was added in a concentration range from 0.01 to 50 µg/ml. After 1 h of incubation, an APC-labeled PD-L1-blocking mAb was added (8 µg/ml) and allowed to compete with the respective test antibodies for PD-L1 or CSPG4 binding for 30 min, after which cell-bound APC levels were quantified by flow cytometry.

CMV-specific T cell stimulation assay

PBMCs derived from CMV-seronegative and CMV-seropositive healthy subjects were cultured in 96-well plates ($1.5x10^5$ cells/well) in the presence of recombinant CMV pp65 protein according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After 96 h, culture supernatants were harvested and stored at -20°C until analyzed for IFN-γ and Granzyme B secretion by ELISA.

Mixed lymphocyte reaction

The capacity of PD-L1xCSPG4 to promote activation and proliferation of T cells was assessed in a mixed lymphocyte reaction (MLR). In short, monocytes were isolated from PBMCs of health subjects by adherence to culture flasks and cultured with IL-4 (500 U/ml) and GM-CSF (800 U/ml). After 3 days, MoDCs were matured for an additional 24 h in the presence of IL-1β (5 µg/ml), IL-6 (15 µg/ml), TNF-α (20 µg/ml), and PGE2 (2.5 mg/ml), essentially as described before (Hobo et al, 2012). For the MLR, CFSE-labeled PBMCs were resuspended in RPMI/10% HS and stimulated with allogeneic MoDC at a cell ratio of 10 to 1. Subsequently, PD-L1xCSPG4 or appropriate control antibodies were added to the wells (5 µg/ml). After 5 days of co-culturing, spent culture medium was assayed for IFN-γ secretion. Subsequently, T cell proliferation was evaluated by CFSE dilution analysis using flow cytometry.

Bioassay for CSPG4-directed PD-1/PD-L1 blockade by PD-L1xCSPG4

Blockade of PD-1/PD-L1 interaction was assessed in a PD-1/PD-L1 Blockade Bioassay (Promega). This assay uses Jurkat-PD1-NFAT-luc T cells expressing PD-1 and NFAT-inducible luciferase and CHO-PD-L1-CD3 cells expressing PD-L1 and a membrane-linked agonistic anti-CD3 antibody. When co-cultured, PD-1/PD-L1 interaction between both cell types inhibits TCR signaling and NFAT-mediated luciferase activity in Jurkat-PD1-NFAT-luc T cells. Addition of a PD-1/PD-L1 blocking agent results NFAT-mediated luciferase activity in Jurkat-PD1-NFAT-luc T cells. The capacity of PD-L1xCSPG4 for CSPG4-directed PD-1/PD-L1 blockade was assessed by replacing CHO.PD-L1/CD3 cells by A375m.EpCAM cells ($CSPG4^{pos}$/$PD\text{-}L1^{pos}$) pretreated with BIS1; an EpCAM-directed CD3-agonistic bsAb. In short, Jurkat-PD1-NFAT-luc T cells were stimulated with BIS1 and mixed with A375m.EpCAM cells at a cell ratio of 5 to 1 and cultured for 18 h in the presence of PD-L1xCSPG4 or appropriate control antibodies. Subsequently, Bio-Glo reagent was added after which bioluminescence was quantified using a Victor V3 multilabel plate reader (Perkin Elmer, Waltham, MA).

IFN-γ ELISpot assay

The enzyme-linked immunospot assay (ELISpot) (Thermo Fisher) was conducted according to the provided protocol. In brief, ELISpot plates (Merck Millipore, Darmstadt, Germany) were coated with anti-IFN-γ antibody and incubated overnight at 4°C. Plates were washed and blocked with DMEM10% FCS for 1 h at RT. A375m, SK-MEL-28 or, FaDu cells were loaded with PD-L1xCSPG4 or control antibodies, after which unbound antibody was washed away. Cancer cells were plated (10.000 cells/well) and freshly isolated PBMCs, stimulated with 0.5µg/ml anti-CD3, were added in a 2:1 or 5:1 ratio to the cancer cells. Plates were

incubated for 48 h at 37°C, washed and coated with detection antibody for 2 h at RT. Next, plates were washed and coated with avidin-peroxidase for 45 min at RT. Plates were washed and developed by addition of aminoethylcarbazole (AEC) substrate. Developed plates were dried and read using an ImmunoSpot reader (AID, Autoimmun Diagnostika GmbH, Strassberg, Germany).

Primary patient-derived melanoma cells and Tumor Infiltrating Lymphocytes

Fresh melanoma tissue was collected during surgical resection after written informed consent (institutional approval by University Medical Center Groningen, nr. METc2012/330). Tissues were minced and short-term cultured in RPMI 1640/10% FCS. Adherent cell phenotype was analyzed by flow cytometry using fluorescently labeled CD14, PD-L1 and CSPG4 antibodies. Primary patient-derived melanoma cells used in this study were $CD14^{neg}$ and $CSPG4^{pos}$ and were used before passage 2. For generation of Tumor Infiltrating Lymphocytes (TILs), minced tissue fragments were cultured in RPMI 1640/10% FCS, supplemented with 50 IU/ml IL-2 (Proleukin, Novartis, Bazel, Swiss). TIL phenotype was confirmed by flow cytometry for CD3, CD4, CD8, PD-1 and PD-L1 expression (Suppl. Fig. 4a-c).

Apoptosis assay

Primary melanoma cells and autologous TILs were mixed in an E:T ratio of 2 to 1, in the presence or absence of PD-L1xCSPG4 or appropriate control antibodies (each 5 µg/ml). At day 3, apoptosis induction in cancer cells (Annexin-V) and CD25 expression on T cells were evaluated by flow cytometry. Spent culture medium was assayed for IFN-γ secretion by an IFN-γ ELISA.

Statistical analysis

Unless otherwise noted, values are mean ± SD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTION

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FIGURE LEGENDS

Figure 1. PD-L1xCSPG4 binds to both PD-L1 and CSPG4 and retains capacity to activate T cells by blocking PD-1/PD-L1 interaction. (**a**) Dose-dependent binding of PD-L1xCSPG4 to MDA-MB-231-CSPG4+++ cells (PD-L1^{pos}/CSPG4^{pos}) versus parental MDA-MB-231 cells (PD-L1^{pos}/CSPG4^{low}). (**b**) Dose-dependent binding of PD-L1xCSPG4 to CHO.PD-L1 cells versus parental CHO cells. (**c**) Binding of PD-L1xCSPG4 (1 μg/ml) to A375m cells in the presence or absence of excess amounts of PD-L1-blocking antibody and/or CSPG4-competing mAb 9.2.27. (**d**) Binding of PD-L1xCSPG4 versus PD-L1xMock (5 μg/ml) to a series of PD-L1^{pos}/CSPG4^{pos} and PD-L1^{pos}/CSPG4^{neg} cancer cell lines. (**e**) Correlation between MFI of PD-L1xCSPG4 binding and MFI of CSPG4 expression of the same cell panel as in d. (**f**) Competitive binding assay in which anti-PD-L1-APC competes with increasing doses (0.01-50 μg/ml) of PD-L1xCSPG4 (black line) or PD-L1xMock (red line) for binding to A375m melanoma cells. Where indicated, A375m cells were pre-treated with excess amounts of mAb 9.2.27 (50 μg/ml) (blue line) or isotype control IgG2a (green line) for 15 min. Graphs a to c represent mean ± SD. R, correlation coefficient.

Figure 2. PD-L1xCSPG4 blocks the PD-1/PD-L1 interaction in an CSPG4-directed manner. (**a**) CSPG4-independent blockade of the PD-1/PD-L1 interaction assessed by a commercially available PD-1/PD-L1 Blockade Bioassay (Promega). Mixed cultures of CHO-K1.PD-L1 cells and Jurkat.PD-1-NFAT-luc cells were treated with increasing doses (0.01-50 μg/ml) of PD-L1xCSPG4, PDL1xMock, MEDI4736 or isotype control. Luciferase activity in the Jurkat cells was quantified using a plate reader. (**b**) Capacity of the indicated test antibodies to dose-dependently (0.64-400 ng/ml) block PD-1/PD-L1 interaction in an CSPG4-directed manner assessed in a modified version of the PD-1/PD-L1 bioassay in which CHO.PD-L1/CD3 cells were replaced by A375m.EpCAM melanoma cells pretreated with

BIS-1; an EpCAM-directed CD3-agonistic bsAb (14). (**c**) Luciferase activity induced by PD-L1xCSPG4 or PD-L1xMock in the presence or absence of excess amounts of CSPG4-competing mAb 9.2.27.

Figure 3. PD-L1xCSPG4 enhances activation status of antigen-experienced T cells. (**a**) Representative histograms of fluorescence dilution of CFSE-labeled PBMCs mixed with allogeneic DCs in an MLR, co-treated with PD-L1xCSPG4 (5 µg/ml) or control antibodies for 5 d. Representative of four independent experiments. (**b**) IFN-γ was measured in four different experiments, performed as in a. (**c**) PBMCs derived from CMV-seropositive or CMV-seronegative subjects were treated with PD-L1xCSPG4 (5 µg/ml) or control antibodies in the presence of CMV protein pp65 for 96 h. IFN-γ or (**d**) Granzyme B levels excreted in culture supernatant were determined by ELISA. Experiments a to d were analyzed by flow cytometry. Graphs c and d represent mean ± SD.

Figure 4. PD-L1xCSPG4 enhances anticancer activity of T cells towards CSPG4^{pos} melanoma cells. (**a**) PBMCs (effector cells) were sub-optimally activated using an agonistic anti-CD3 antibody and then added to the A375m target cells at an E:T cell ratio of 2:1. PD-L1xCSPG4 (5 µg/ml) or control antibodies were added to the wells. IFN-γ ELISpots per 20.000 PBMCs corrected for medium control. (**b**) Photos showing IFN-γ ELISpots produced during mixed culture of PBMC with A375m cells (CSPG4^{pos}), as described in a. (**c**) Cancer cells were treated with PD-L1xCSPG4 (5 µg/ml) or control antibodies at 4°C for 1h, after which unbound antibody was removed. PBMCs were activated using an agonistic anti-CD3 antibody and then added to the cancer cells in an E:T cell ratio of 2:1 or 5:1. A375m cells (CSPG4^{pos}), SK-MEL-28 cells (CSPG4^{pos}) or FaDu (CSPG4^{neg}) cells treated with PD-L1xMock or PD-L1xCSPG4.

Figure 5. PD-L1xCSPG4 enhances anticancer activity of TILs towards autologous patient-derived melanoma cells. Histograms showing cell surface expression of (**a**) CSPG4 (red) and (**b**) PD-L1 (red) on primary melanoma cells derived from 5 individual melanoma patients. Binding of isotype control antibody is shown in black. TILs derived from 5 melanoma patients were cocultured with corresponding autologous melanoma cells in an E:T ratio of 2:1 and treated with PD-L1xCSPG4, or control antibodies (5µg/ml). The percentage of apoptotic cancer cell death (**c**) and the percentage of CD25 expression (**d**) on TILs were determined by flow cytometry. (**e**) IFN-γ levels in culture supernatant of C as detected by ELISA. MFI, mean fluorescence intensity.

Fig. 1

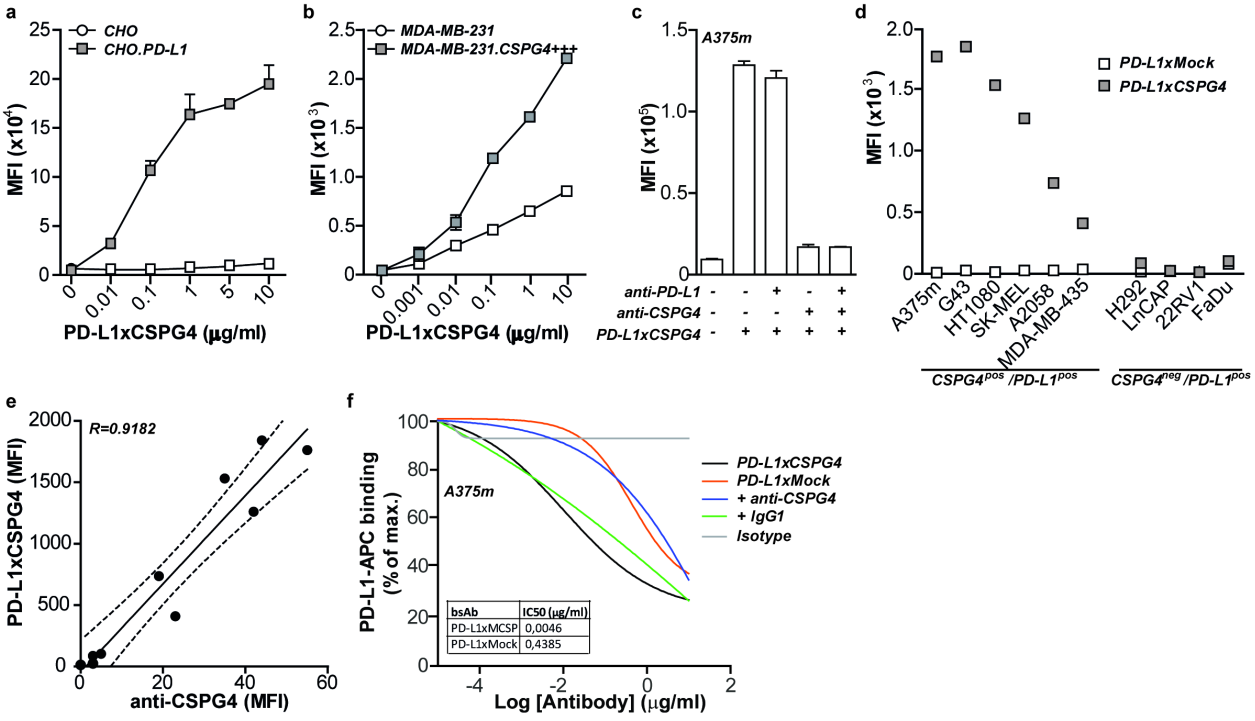
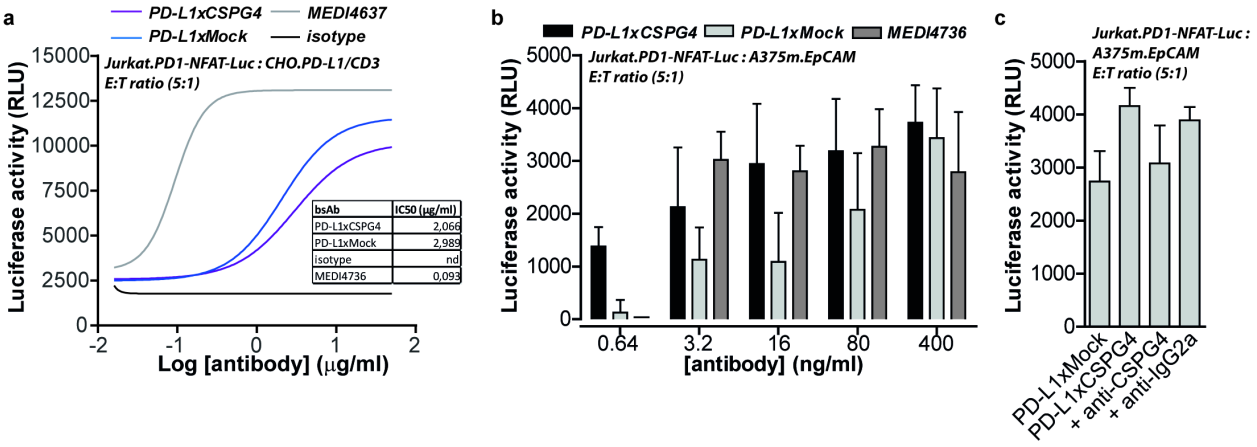

a
CHO
CHO.PD-L1
MFI (x10⁴)
PD-L1xCSPG4 (µg/ml)
b
MDA-MB-231
MDA-MB-231.CSPG4+++
MFI (x10³)
PD-L1xCSPG4 (µg/ml)
c
A375m
MFI (x10⁵)
anti-PD-L1
anti-CSPG4
PD-L1xCSPG4
d
PD-L1xMock
PD-L1xCSPG4
MFI (x10³)
A375m
G43
HT1080
SK-MEL
A2058
MDA-MB-435
H292
LnCAP
22RV1
FaDu
CSPG4pos /PD-L1pos
CSPG4neg /PD-L1pos
e
R=0.9182
PD-L1xCSPG4 (MFI)
anti-CSPG4 (MFI)
f
A375m
PD-L1-APC binding (% of max.)
Log [Antibody] (µg/ml)
PD-L1xCSPG4
PD-L1xMock
+ anti-CSPG4
+ IgG1
Isotype
bsAb | IC50 (µg/ml)
PD-L1xMCSP | 0,0046
PD-L1xMock | 0,4385

Fig. 2



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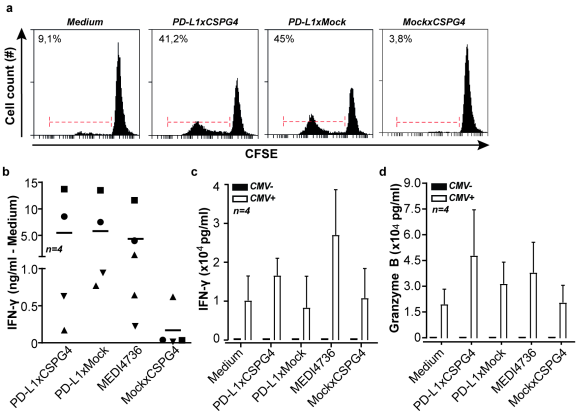

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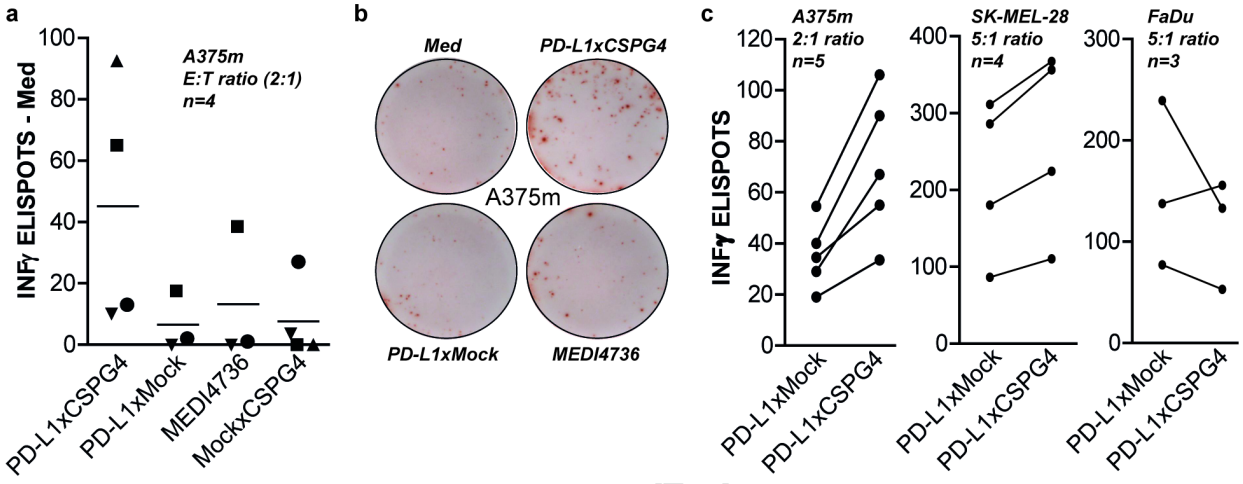

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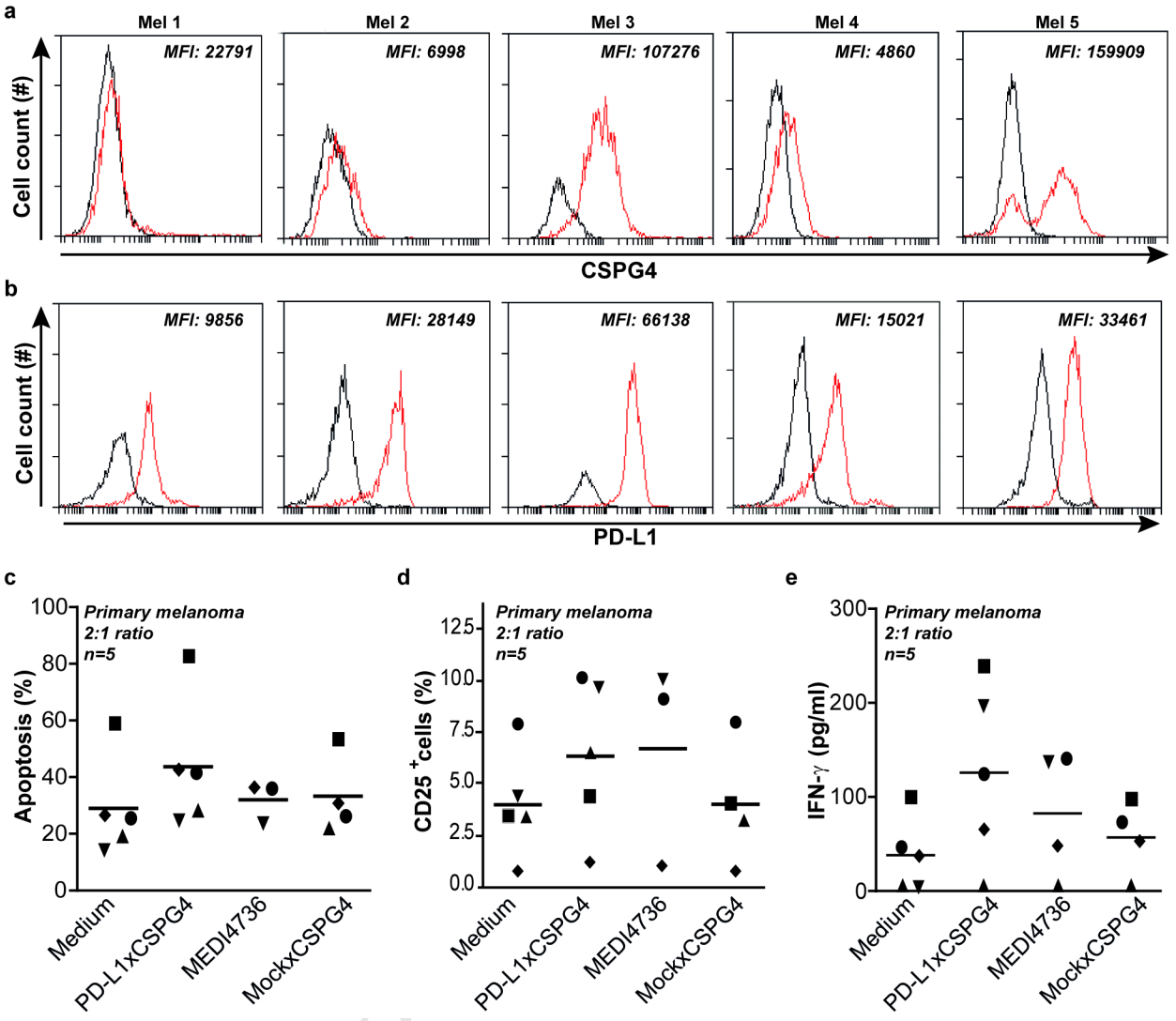


SUPPLEMENTARY TEXT

Suppl. Fig 1. Construction and production of PD-L1xCSPG4. (**a**) A eukaryotic expression plasmid pEE14-bsAb, which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 IgG domain. MCS#1, MCS#2 and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFvCSPG4, and human IgG1 Fc domain, respectively, yielding plasmid pEE14-PD-L1xCSPG4. DNA fragments encoding scFvPD-L1 and scFv9.2.27 were generated by commercial gene synthesis service (Genscript, Piscataway, NJ) based on published VH and VL sequence data of PD-L1-blocking antibody 3G10 and CSPG4-directed mAb 9.2.27 from patents US20110209230A1 and US20050244416A1, respectively. (**b**) Schematic representation of PD-L1xCSPG4. scFvPD-L1 (light blue); scFvCSPG4 (red); human Fc-IgG1 (dark blue). (**c**) SDS-PAGE analysis of PD-L1xCSPG4. Protein A-purified MEDI4736 (lane 1 and 2) or PD-L1xCSPG4 (lane 3 and 4) (2,5µg/ml each) were separated by an SDS-PAGE gel with or without reduction. Under non-reducing conditions PD-L1xCSPG4 has an apparent molecular weight of 175 kDa (lane 3), which dropped to 80 kDa under reducing conditions (lane 4). MEDI4736 showed the expected heterodimeric composition of heavy and light chain characteristic for conventional antibodies (lane 1 and 2). M, marker. NR, non-reduced. R, reduced.

Suppl. Fig 2. Correlation PD-L1xCSPG4 and PD-L1xMock binding and CSPG4 and PD-L1 cell surface expression. (**a**) Correlation between the MFI of PD-L1xMock binding and the MFI of CSPG4 expression. (**b**) Correlation between the MFI of PD-L1xCSPG4 binding and the MFI of PD-L1 expression. (**c**) Correlation between the MFI of PD-L1xMock binding and the MFI of PD-L1 expression. (a) to (c) were performed on the same cell panel as in **Fig.1e**. R, correlation coefficient.

Suppl. Fig 3a. PD-L1xCSPG4 simultaneously binds to CSPG4 on one cell type and PD-L1 on a nearby other cell type forming cell clusters. Representative flow cytometer dot-plots demonstrating that bsAb PD-L1xCSPG4 can simultaneously bind and cellularly bridge (DiD-labeled) $CSPG^{high}$ A375m melanoma cells and (CSFE-labeled) $PD\text{-}L1^{high}$ CHO.PD-L1 cells as is evident from a marked increase in DiD/CFSE double-positive events. This aggregation is fully abrogated in the presence of a CSPG4-blocking antibody.

Suppl. Fig. 3b. Percentage DiD/CSFE double-positive events upon incubation with indicated antibodies. Control bsAb PD-L1xMock antibody equipped with an irrelevant second binding specificity, as well as the clinically used monospecific PD-L1-blocking antibody MEDI4736 (durvalumab), failed to enhance clustering of both cell types (black bars). Additionally, when the experiment was performed using PD-L1-negative parental CHO cells instead of CHO.PD-L1 cells, no enhanced cluster formation was observed (gray bars).

Suppl. Fig 4. Marker expression on tumor-infiltrating lymphocytes. (**a**) TIL's were stained with anti-CD3 and anti-CD8 antibodies. Percentages of T cells are presented in the plot. Percentage (**b**) and MFI (**c**) of PD-1 and PD-L1 expression on gated CD3 T cells. Data are representatives of two donors. MFI, mean fluorescence intensity.

Suppl. Fig 5. Assessment of CSPG4 cell surface expression using secondary signal amplification. (a) CSPG4 expression level on A2058 melanoma cells as detected by using a primary FITC-conjugated anti-CSPG4 mAb only (one-step method) compared to **(b)** CSPG4 expression level detected after subsequent signal amplification using alexafluor647 labeled secondary anti-mouse polyclonal antibody preparation (two-step method). **(c)** CSPG4

expression level on primary patient-derived melanoma cells (MEL6) as detected by using a primary FITC-conjugated anti-CSPG4 mAb only (one-step method) compared to **(d)** CSPG4 expression level detected after subsequent signal amplification using alexafluor647-labeled secondary anti-mouse polyclonal antibody preparation (two-step method).

Supplementary Table I

Relative indexes of cell surface expression of CSPG4 and PD-L1 by a series of cancer cell lines by flow cytometry using anti-CSPG4-FITC and anti-PD-L1-APC antibodies, respectively.

Cell lines	CSPG4	PD-L1
A375m	++++	+
G43	+++	+
HT1080	+++	++
SK-MEL-28	+++	+++
A2058	++	+
MDA-MB-231	+	++++
MDA-MB-231.CSPG4+++	++++	++++
MDA-MB-435	+	+
H292	-	+++
LNCaP	-	++
22RV1	-	+
FADU	-	++++

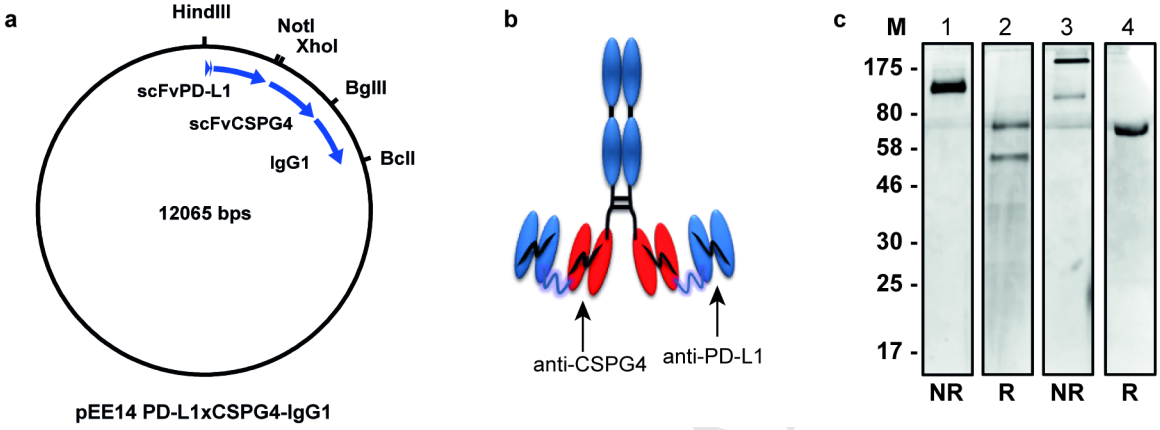
relative expression index of CSPG4:

\+ = MFI <10
++ = MFI 10-30
+++ = MFI 30-50
++++ = MFI >50

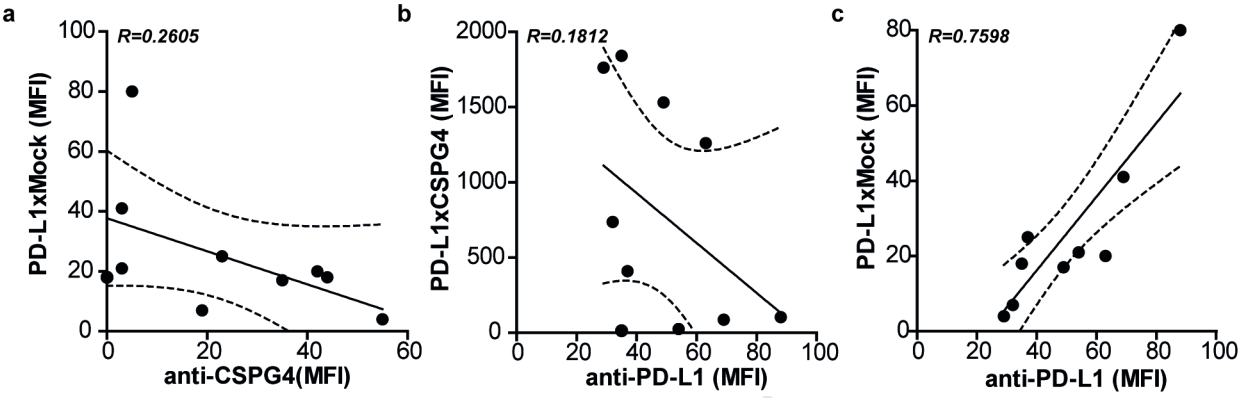
relative expression index of PD-L1:

\+ = MFI <40
++ = MFI 40-60
+++ = MFI 60-80
++++ = MFI >80

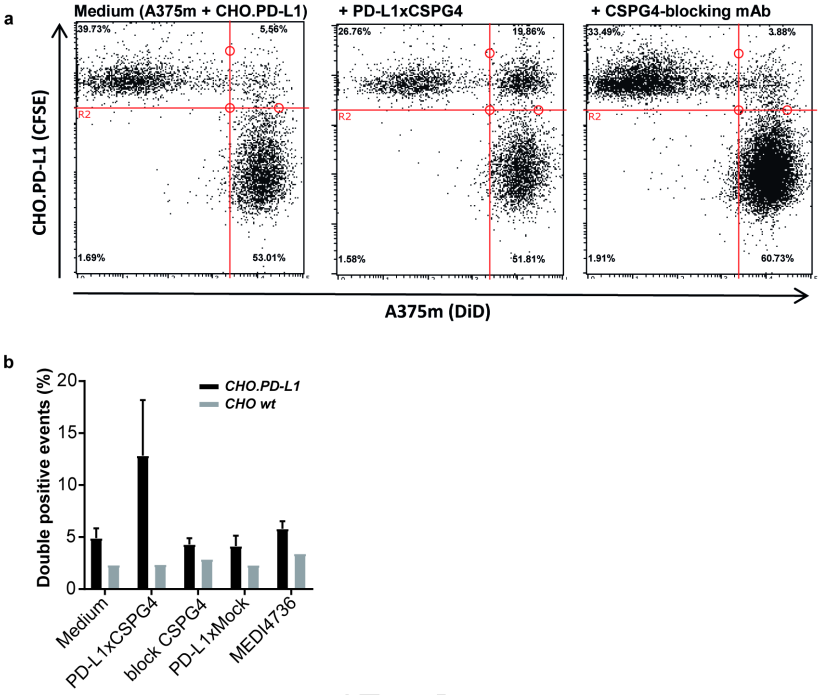
Supplementary Fig. 1



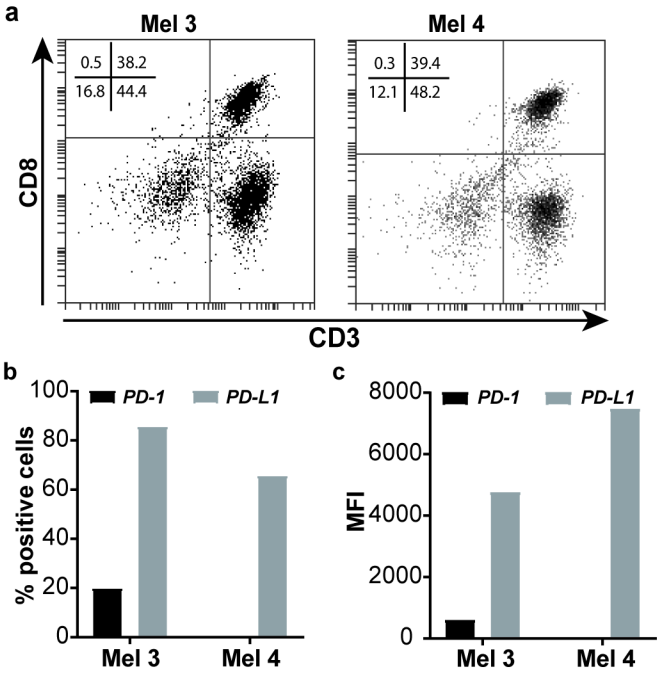
Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4



Supplementary Fig. 5